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Anthocyanin Biosynthetic Genes and Their Application to Flower Color Modification through Sense Suppression

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Flower color of important commercial cut flower crops is determined principally by two pigment types: anthocyanins and carotenoids. The former pigments give rise to orange, pink, red, purple, and blue, whereas the latter are principally responsible for a range of yellow and orange colors. The biosynthesis of anthocyanin pigments and the factors responsible for determining flower color from these pigments have been studied extensively (Forkmann, 1991). A good molecular genetic understanding of anthocyanin biosynthesis has been developed for snapdragon (*Antirrhinum majus* L.), petunia (*Petunia ×hybrida* Hort.), and corn (*Zea mays* L.). This understanding has made it possible to modify flower color of cut flower crops in a commercially meaningful way.

There are two reasons to modify flower color commercially. First, the flower color of a variety with desirable agronomic or consumer characteristics can be modified to another flower color typical of that crop. For example, a white-flowering carnation (*Dianthus caryophyllus* L.) could be created from a red-flowering variety with highly desirable agronomic or consumer characteristics. Second, a flower color that does not occur naturally in a particular crop can be introduced into that crop. For example, a blue-flowering rose (*Rosa hybrida* L.) variety could be developed through the introduction of petunia genes whose products interact to produce blue pigmentation.

To achieve these ends, either the expression of host plant genes must be suppressed or nonhost plant genes must be expressed. The introduced expression of nonhost plant genes can have unpredictable effects on flower color, and the final color depends on the precise biochemistry of anthocyanin biosynthesis in a particular variety. However, the range of flower colors resulting from suppression of an anthocyanin pathway gene is fairly predictable.

I will focus here on the suppression of the first committed step in anthocyanin biosynthesis—chalcone synthase (see Fig. 1 for outline of the pathway). Completely white-flowering plants have been produced from red- or purple-flowering plants through sense suppression of chalcone synthase expression in petunia (Napoli et al., 1990; van der Krol et al., 1990). This example will serve to illustrate some of the issues involved in modifying flower color through genetic engineering. It also will illustrate the practical application of flower color modification in chrysanthemum [*Dendranthema ×grandiflorum* (Ramat.) Kitamura], carnation, and rose.

ISOLATION OF CHALCONE SYNTHASE SEQUENCES

The first chalcone synthase (CHS) gene to be cloned was isolated from parsley [*Petroselinum crispum* (Mill.) Nym. ex A.W. Hill] (Kreuzaler et al., 1983) by using an immunological approach. The maize *c2* gene, encoding a CHS gene expressed in the aleurone layer of the kernel, was isolated subsequently, using transposon tagging (Wienand et al., 1986). Additional CHS genes have been isolated using hybridization to cDNA libraries or genomic libraries with the previously isolated CHS clones. The snapdragon and petunia genes were isolated in this way using the parsley clone as a heterologous probe (Reif et al., 1985; Spribille and Forkmann, 1982). Similar approaches have been taken to isolate other CHS genes of use for color modification [for example, the CHS genes of chrysanthemum (Courtney-Gutterson et al., 1994), carnation (unpublished data), and gerbera (*Gerbera jamesonii* H. Bolus ex Hooker) (Elomaa et al., 1993)]. The high sequence conservation of the CHS genes from various plant families (Niesbach-Klosgen et al., 1987) has enabled this approach to be carried out with little difficulty.

Once a particular pathway gene has been isolated from one or more plants, the hybridization approach can be taken to isolate a homologous gene from other plant sources. The utility of this hybridization approach to gene isolation depends on relative sequence conservation. Even for a well-conserved pathway, such as anthocyanin biosynthesis, there is significant variation in sequence conservation among various genes of the pathway. For example, CHS sequences are more highly conserved than dihydroflavonol reductase (DFR) sequences, which, in turn, are more highly conserved than chalcone isomerase (CHI) sequences, as illustrated by the percent similarities and identities (Table 1) for amino acid and nucleotide sequences of the relevant coding regions for three species in which all three gene sequences are known. Consequently, isolating a CHI gene by hybridization using an intact coding sequence is generally more difficult than isolating a CHS gene. Coding sequences from evolutionarily related plants are more conserved, so they can be more useful for gene isolation purposes [e.g., a pea (*Pisum sativum* L.) CHI clone would be a more useful probe for hybridization to an alfalfa (*Medicago sativa* L.) library than to a petunia library; see CHI nucleotide sequence comparisons in Table 2].

When a gene is needed from a plant for which no sufficiently related sequences have been isolated previously, a polymerase chain reaction (PCR) approach is possible. Conserved amino acid sequences of this gene can be found by comparing all of the available sequences, and degenerate PCR primers can be derived from the conserved region. Once isolated, such a cDNA fragment can be used to identify full-length cDNA clones by hybridization, or to reduce expression of the host gene through sense suppression. For example, PCR amplification has been used to isolate a partial CHS cDNA clone of rose, using primers designed to recognize highly conserved dicot CHS sequences (see below); this clone has been used to suppress CHS expression.

Table 1. Sequence comparisons for anthocyanin pathway genes for *Arabidopsis thaliana* and *Petunia ×hybrida*.[z]

Plant 1	Plant 2	CHS[y]	CHI[y]	DFR[y]
A. thaliana	*P. ×hybrida*	92.8[x]	74.7	75.1
		85.1[w]	59.1	63.2
		70.2[v]	60.3	62.5
	Antirrhinum majus	92.0	70.7	77.5
		84.8	57.2	63.1
		72.0	63.1	62.6
P. hybrida	*A. majus*	95.6	76.3	83.4
		90.2	62.3	70.5
		76.5	64.9	71.8

[z]The coding sequence is compared for each pair of genes. Comparisons were done using the gap program of the Wisconsin GCG sequence analysis package.
[y]CHS = chalcone synthase; CHI = chalcone isomerase; DFR = dihydroflavonol reductase.
[x]First row for each plant pair presents the percent amino acid similarity.
[w]Second row for each plant pair presents the percent amino acid identity.
[v]Third row for each plant pair presents the percent nucleotide sequence identity.

Table 2. Nucleotide sequence comparisons for chalcone isomerase genes.[u]

	Pisum sativum	*Petunia ×hybrida*	*Phaseolus vulgaris*	*Medicago sativa*	*Malus domestica*	*Arabidopsis thaliana*
A. majus	59.1	64.9	61.8	61.7	66.2	63.1
A. thaliana	53.9	60.3	57.6	55.2	65.8	
M. domestica	59.7	67.3	59.9	62.0		
M. sativa	82.4	58.0	80.0			
P. vulgaris	75.2	60.5				
P. ×hybrida	57.9					

[u]The nucleotide sequence, over the protein coding region, is compared for each gene combination. Numbers are percent sequence identity. Comparisons were done using the gap program of the Wisconsin GCG sequence analysis package.

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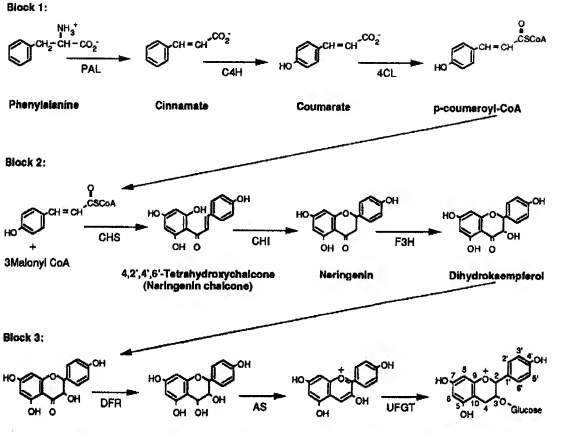

Fig. 1. Biosynthetic pathway for the anthocyanin pelargonidin-3-glucoside, which illustrates the core steps for synthesis of all anthocyanins. Block 1 includes the reactions of the general phenylpropanoid pathway; block 2 includes the reactions of the general flavonoid pathway; block 3 includes reactions specific for anthocyanin biosynthesis. PAL = phenylalanine ammonia lyase; C4H = cinnamate 4-hydroxylase; 4CL = 4-coumarate ligase; CHS = chalcone synthase; CHI = chalcone isomerase; F3H = flavanone 3-hydroxylase; DFR = dihydroflavonol reductase; AS = anthocyanidin synthase; UFGT = UDPG flavonoid 3-glucosyl transferase.

SUPPRESSION OF ANTHOCYANIN BIOSYNTHESIS IN PETUNIA

Importance of related sequences in sense suppression

Homologous and heterologous sequences have been compared for their capacity to sense-suppress endogenous gene expression in petunia. Although heterologous sequences can be used for sense suppression, a high degree of sequence identity was needed. Separate sense gene constructs were made with petunia CHS and DFR coding sequences and chrysanthemum CHS and DFR coding sequences; both of the chrysanthemum sequences are 70% identical to the petunia sequences. The CaMV 35S promoter was used to program expression in each case, with a standard nopaline synthase 3′ sequence added. The T-DNA elements carry a *35S-nptII* marker gene in addition to the specific suppressing construct.

Using the petunia CHS sequence, eight of 48 independent transformants of petunia inbred line V26 were suppressed for anthocyanin biosynthesis. In contrast, 0 of 97 independent V26 petunia plants transformed with chrysanthemum CHS were suppressed ($P \leq 0.001$; chi-square). Similar data were obtained for the petunia and chrysanthemum DFR coding sequences (petunia: 9 of 91; chrysanthemum: 0 of 35; $P = 0.15$, by chi-square). Although the difference in frequency of suppression is not statistically significant for the DFR comparison, the same trend is observed in that no suppression was found with the heterologous sequence.

In petunia, two CHS genes are expressed in corolla tissue: CHS-A, responsible for 90% of CHS mRNA accumulation, and CHS-J, responsible for 10% of CHS mRNA accumulation. In white-flowering plants obtained using a CHS-A coding sequence in the suppressing transgene, both CHS-A and CHS-J have been suppressed (Morgan and Gutterson, unpublished data). These two genes share 85% sequence identity throughout the coding regions. Thus, complete identity is not required for suppression. Nonetheless, because the extent of sequence identity cannot be determined without cloning at least a portion of the endogenous gene of the host plant, it is sound practice to isolate the host gene and to carry out sense suppression with the host gene sequence.

Utility of partial gene sequences

The ease of isolating partial gene sequences via the polymerase chain reaction prompted an analysis of the utility of gene fragments in sense suppression. If small DNA fragments could be used, simplicity of the suppression approach would be improved. To test this, we used the petunia CHS system again, working with the inbred line V26, and using portions of the CHS-J coding sequence in transgenes. The full-length sequence of ≈1150 bp was compared with a 550-bp fragment located in the latter two-thirds of the gene, and with subfragments of either 225 bp or 125 bp. CHS-A is responsible for ≈90% of CHS expression in V26; thus, suppression of both CHS-A and CHS-J is presumably necessary to generate plants producing completely white flowers. We observed suppression at sizes down to 225 bp; however, the proportion of plants with a high level of suppression decreased with decreasing fragment length, most notably below 550 bp. These data indicate that gene fragments can be used, but that fragments longer than 600 bp are preferred for a good frequency of plants with effective suppression. Others have used gene fragments to sense-suppress plant genes (Dunsmuir et al., personal communication; Smith et al., 1990).

SENSE SUPPRESSION OF CHS IN FLOWER CROPS

Sense suppression of CHS to produce either white- or paler-flowering derivatives of pink- or red-flowering parents has been achieved in chrysanthemum, carnation, and rose. In chrysanthemum, white-flowering derivatives were produced; in carnation and rose, only partial suppression of anthocyanin biosynthesis was achieved.

Chrysanthemum

Introduction of either sense or antisense chimeric gene constructs (using a full-length chrysanthemum coding sequence) into the pink-flowering chrysanthemum cultivar Moneymaker resulted in a small proportion of transformants with white-flowering plants (Courtney-Gutterson et al., 1994). The frequency of suppressed individuals in the transformant population was similar for the sense and antisense constructs. Some white-flowering transgenics were tested and found to be suppressed for CHS expression by RNAse protection analysis. Good stability of the white-flowering phenotype was found for a single anti-sense- and a single sense-suppressed line when propagated vegetatively for several "generations." Stability was similar for the sense and antisense plants.

Carnation

A chimeric sense construct, produced using a full-length carnation CHS cDNA clone, was introduced into the pink-flowering carnation cultivar Manon. About 10% of more than 100 transgenic plants containing this construct had reduced anthocyanin pigmentation compared with the parental cultivar. A range of flower color intensity was observed among the suppressed plants, with uniform flower color throughout petal tissue. This result illustrates the quantitative nature of sense suppression. Flower color is currently being monitored (by scientists at Florigene Europe) in successive cutting generations.

Rose

A rose CHS cDNA clone of ≈800 bp was isolated using PCR amplification from rose floral RNA. A pair of nondegenerate primers was designed based on sequences highly conserved among dicot CHS genes. A chimeric gene was constructed and introduced into the rose cultivar Royalty, which produces dark-red flowers. Of the 110 transformants produced, 15 had uniformly reduced anthocyanin pigmentation. Since a fragment of the CHS gene was used, accumulation of transgene and endogenous gene messages was monitored using Northern hybridization. In most cases, substantial levels of transgene message were detected, with significantly reduced accumulation of endogenous gene message compared to controls. Primary transformants have been maintained now for more than 18 months; in some cases, the extent of suppression increased at first and then stabilized, and in other cases, suppression has been stable throughout.

CONCLUSION

The utility of sense suppression of anthocyanin biosynthetic genes to modify flower color has been demonstrated with chalcone synthase. This approach is fairly predictable, with a range of possible flower colors being produced due to the quantitative nature of suppression. Because virtually all of the main anthocyanin biosynthetic pathway genes have been isolated now from more than one plant source, broad application of this approach is possible. It should be possible to identify an appropriate gene for a specific color change, to isolate the gene based on sequence conservation, and to produce plants altered for expression of the gene and flower color. This approach to modifying flower color offers a useful alternative, or adjunct, to conventional breeding.

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